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Award Number: W81XWH-08-2-0126

TITLE: Developing Memory Reconsolidation Blockers as Novel PTSD Treatments

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REPORT DATE: June 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                           Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

    x Approved for public release; distribution unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
30-06-2009	Annual	1 June 2008-31 May 2009

4. TITLE AND SUBTITLE
Developing Memory Reconsolidation Blockers as Novel PTSD Treatments

5a. CONTRACT NUMBER
W81XWH-08-2-0126

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The General Hospital Corporation
Boston, MA, 02114

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We have successfully implemented the proposed animal experiments at each study site. Key accomplishments to date are the demonstration that the anti-progesterone and glucocorticoid receptor antagonist mifepristone, and the protein-synthesis inhibitor rapamycin, when administered systemically reduce reconsolidation of a cue-conditioned fear response in rats. We have also made substantial progress toward implementing the first two studies we will undertake in human subjects. First, a double-blind, randomized, placebo-controlled pilot trial of six sessions of post-reactivation propranolol for the treatment of PTSD is in the final stages of obtaining DOD IRB approval, after which recruitment will begin. Second, we have formulated the design of a pilot study of single-session, post-reactivation mifepristone's ability to reduce psychophysiologic responses during traumatic imagery in trauma-exposed human subjects. We have succeeded in obtaining an investigational new drug (IND) approval from the FDA for this post-marketing application of mifepristone. We are now in the final stages of obtaining approval from the local institutional review board (IRB). Following that, we will prepare a DOD IRB submission.

15. SUBJECT TERMS
Stress disorders, post-traumatic; reconsolidation; imagery, psychotherapy; pharmacology; psychophysiology (all MeSH terms)

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	12	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. INTRODUCTION

The aim of this project is to develop post-reactivation (PR) pharmacologic interventions that may serve as novel treatments for posttraumatic stress disorder (PTSD). The underlying theory is that candidate drugs, when given following the reactivation of a conditioned fear response in animals, or a traumatic memory in humans, will reduce the strength of the conditioned response or traumatic memory. We plan to test such drugs, either alone or in combination, for their possible reconsolidation-blocking properties in a hierarchy of experiments. Drugs that show promise at a given stage of investigation will be advanced to the next stage. In Stage I, we will evaluate the ability of candidate drugs to reduce freezing in a Pavlovian cue-conditioned fear task in rats, as well as to reduce associated retrieval-induced activation of immediate early genes in the amygdala. In Stage II, we will evaluate the ability of candidate drugs to reverse fear conditioning-induced synaptic enhancement in rat amygdala slices using whole-cell electrophysiologic recording. In Stage III, we will test the ability of a single session of PR candidate drug to reduce subsequent psychophysiologic responding during script-driven imagery of the traumatic event in trauma-exposed human subjects. In Stage IV, we will test the ability of multiple PR candidate drug therapy sessions to reduce symptoms in PTSD patients.

The animal reconsolidation experiments will entail three phases: 1.) single-trial fear conditioning; 2.) presenting the conditioned stimulus (reactivation), followed by PR drug; and 3.) measuring the conditioned response in a test trial, followed by sacrificing the animal for immunohistochemical or electrophysiologic measurements. If the drug is an amnestic (i.e., reconsolidation-blocking) agent, the test conditioned response should be reduced in animals that previously received the drug. Because the (past) traumatic event itself represents the (phase 1) conditioning event, the human experiments will only have the last two stages: 2.) single or multiple sessions of traumatic memory reactivation followed by candidate drug; and 3.) measuring a.) psychophysiologic responses during script-driven imagery of the traumatic event, and/or b.) PTSD symptoms.

In order to rule out the possibility that nonspecific drug effects account for any findings, the experiments will incorporate non-reactivation (NR) drug control groups, as well as PR vehicle/placebo control groups

2. BODY

2.1. Animal work

### 2.1.1. Massachusetts General Hospital (MGH)

2.1.1.1. <u>1.5 mV unconditioned stimulus (UCS).</u> We initially performed a series of experiments with this higher UCS stimulus intensity. On Day 1, rats were conditioned by pairing a single tone conditioned stimulus (CS) with a single shock UCS. On Day 2, rats were exposed to a single CS trial, and the conditioned response (CR, i.e., duration of freezing) was measured. This was immediately followed by i.p. injection of PR drug or vehicle. Alternately, rats received the nonreactivated (NR) drug injection in the absence of the CS trial.   On Day 3 and again on Day 10, rats were exposed to a single CS trial, and the CR was measured. All Day 1, 2, 3, and 10 procedures were performed in the same context. Results obtained with various drugs in male rats are displayed in MGH Figure 1. Significant findings were confined to PR mifepristone, an anti-progesterone and glucocorticoid receptor antagonist. This drug produced significant

reconsolidation blockade in comparison to both PR vehicle (Day 3 test p=0.001, Day 10 test p=0.003) and NR mifepristone (Day 3 test p=0.02, Day 10 test p=0.008). Paradoxically, either propranolol or ondansetron reversed this effect. No significant results were obtained for PR mifepristone in male rats, or for any drug in female rats, which were more variable in their responses than males.
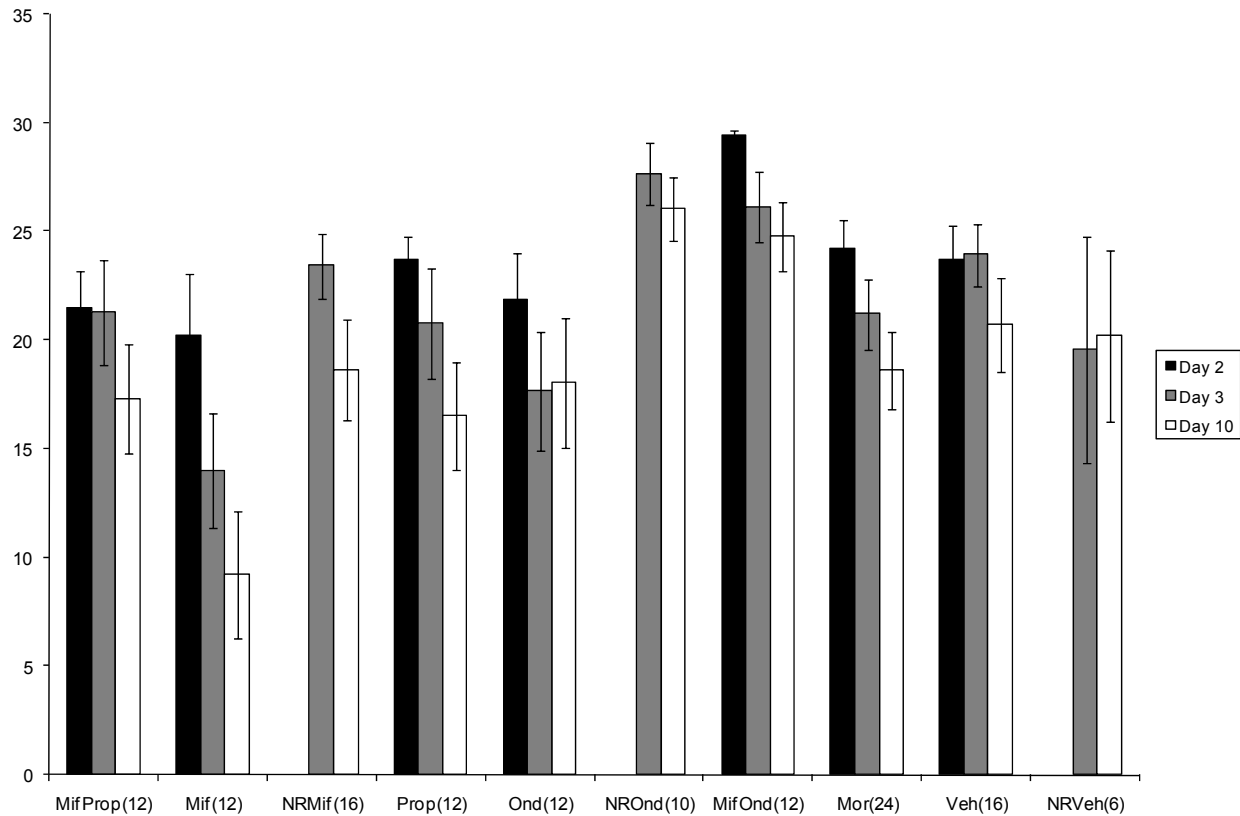
MGH Figure 1. **Results at the 1.5 mV UCS shock intensity with various drugs (male rats).** The y-axis indicates the strength of the CR in seconds of freezing (maximum 30 sec). Errors bars indicate SEM. Key: MifProp: mifepristone (30 mg/kg) plus propranolol (10 mg/kg); Mif: mifepristone (30 mg/kg); NRMif: non-reactivated mifepristone (30 mg/kg); Prop: propranolol (10 mg/kg); Ond: ondansetron (0.2 mg/kg); NROnd: non-reactivated (ondansetron 0.2 mg/kg); MifOnd: mifepristone (30 mg/kg) plus ondansetron (0.2 mg/kg): Mor: morphine sulfate (1 mg/kg); Veh: vehicle; NRVeh: non-reactivated vehicle. Numbers of rats are indicated in parentheses.

2.1.1.2. 0.75 mV unconditioned stimulus (UCS). It is possible that at the higher UCS intensity, CRs are more resistant to reconsolidation blockade, making it more difficult to identify drug effects. In consideration of this possibility, we have recently switched to this lower UCS intensity. So far we have performed experiments with PR mifepristone. This produced significant reconsolidation blockade in comparison to both vehicle (Day 3 test p<0.001, Day 10 test p=ns) and NR mifepristone (Day 3 test p=0.001, Day 10 test p=0.01) in male and female rats combined (MGH Figure 2), as well as in each gender separately.

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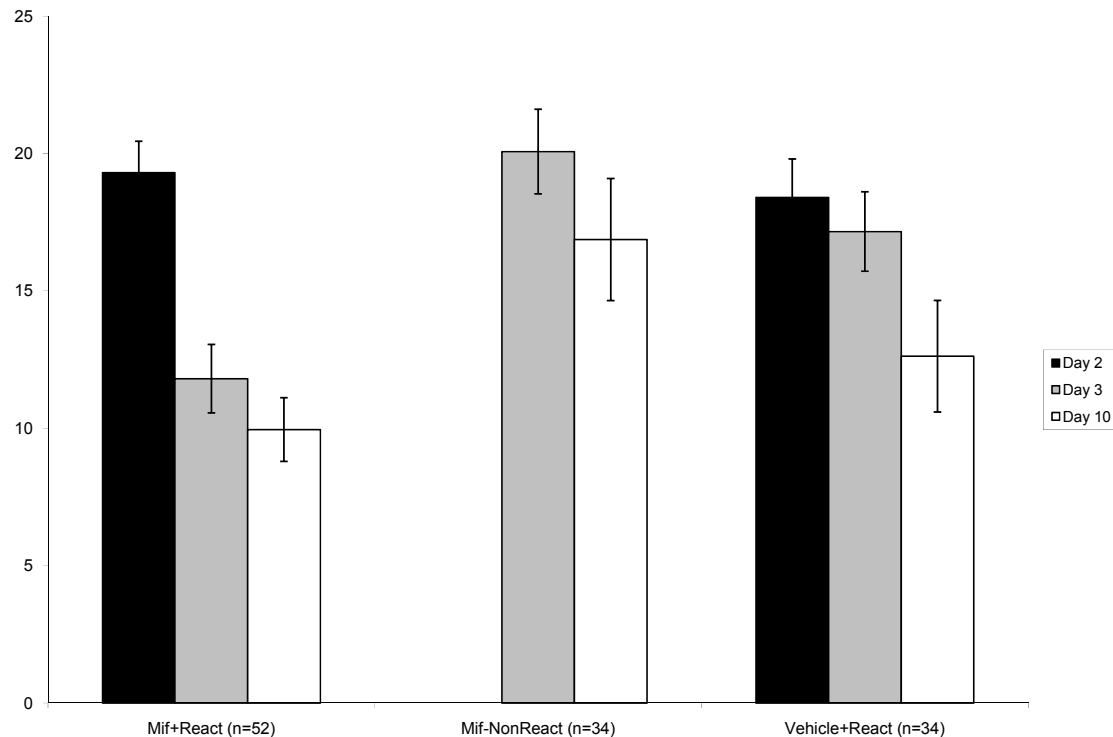
MGH Figure 2. **Results at the 0.75 mV UCS shock intensity with mifepristone (males and female rats combined).** The y-axis indicates the strength of the CR in seconds of freezing (maximum 30 sec). Errors bars indicate SEM. Numbers of rats are indicated in parentheses.

2.1.2. McGill University. To date, we have investigated the reconsolidation blockade of auditory fear memories using various doses (5, 10, 20, 40mg/kg) of the beta-adrenergic blocker, propranolol, injected immediately after the reactivation session (McGill Figure 1A). To minimize the possible effect of stress on the treatment, we also injected propranolol (2 or 10 mg/kg) at different time points on the reactivation day: either 20 minutes prior or 2 hours after the reactivation session (McGill Figure 1B). Our initial results revealed an inability of propranolol to block reconsolidation under the parameters tested. Following these unexpected results, we tried injecting propranolol (10mg/kg) after multiple reactivation sessions to mimic the treatment in patients. Again, this protocol did not show reconsolidation blockade of the fear-related memory (McGill Figure 1C). As neither changing drug concentration nor injection time point were successful in impairing reconsolidation of auditory fear memory, it seems fair to conclude that propranolol might not be effective in the specific task we used here. Therefore, we decided to explore another candidate drug, mifepristone.

Mifepristone was injected 10 or 30 mg/kg immediately after reactivation of the conditioned fear memory. Although the 30 mg/kg dose slightly reduced the CR, the fear response of these animals was not significantly different from the vehicle-injected controls (McGill Figure 2B). To further determine mifepristone's efficacy in disrupting memory reconsolidation, we trained rats with lower shock intensity (McGill Figure 2C) or waited one week after training before

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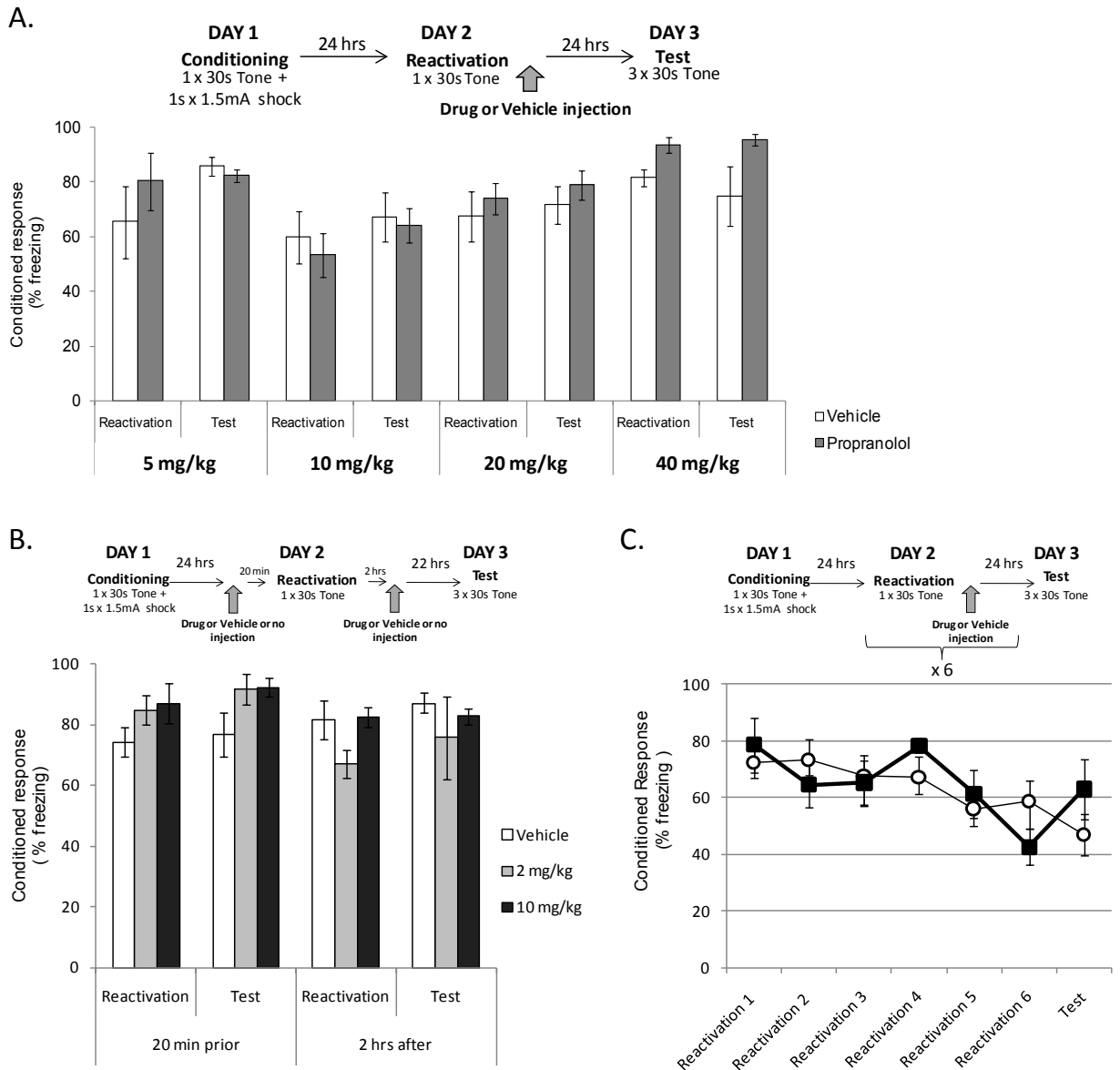
Figure 1. Propranolol does not disrupt reconsolidation of auditory fear memories. A) At the doses of 5, 10, 20 or 40 mg/kg (respectively n=4, n=12, n=10, n=4), propranolol did not significantly impair reconsolidation injected immediately after the reactivation session compared to the vehicle group (respectively n=4, n=12, n=10, n=4). B) Injecting propranolol either 20 minutes before or 2 hours after reactivation did not impair reconsolidation of the conditioned fear memory. When injected 20 minutes prior to reactivation, a 2 mg/kg (n=4) or a 10 mg/kg (n=8) dose a propranolol did not decrease the conditioned response compared to the vehicle group (n=8). Similar results are found when the propranolol injection is performed 2 hours after the reactivation session (vehicle, n=8; 2 mg/kg, n=4; 10mg/kg, n=8). C) Propranolol failed to block memory reconsolidation after multiple treatments and reactivation sessions. Rats received 6 reactivation sessions followed by an injection of propranolol (10 mg/kg, squares; n=8). The propranolol-treated rats did not show a decreased conditioned response compared to the vehicle-treated rats (circles; n=8) for any of the reactivation sessions or on the test day.
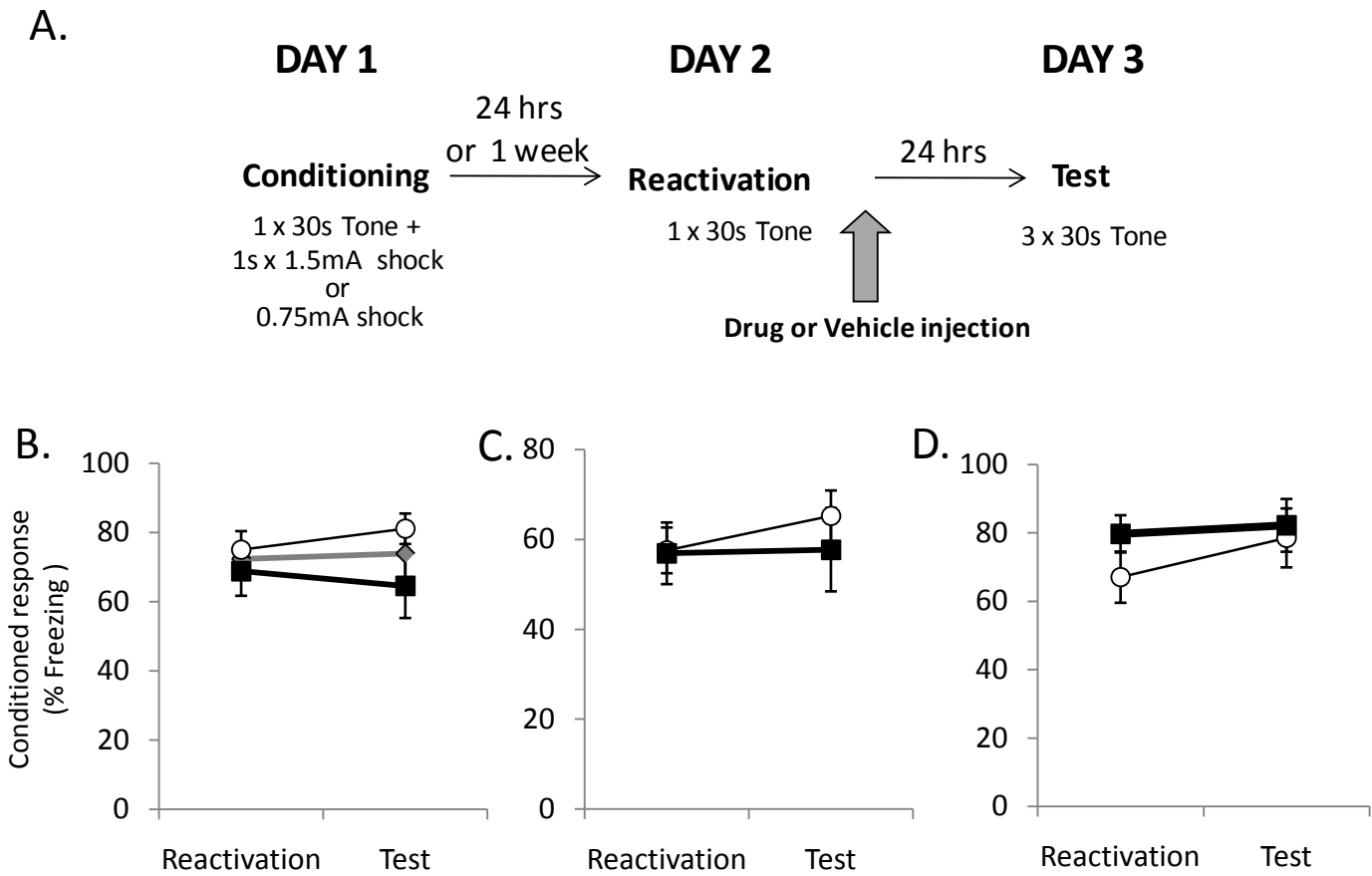
Figure 2. Mifepristone does not block reconsolidation of auditory fear memories. A) Experimental protocol. B) Mifepristone 10 mg/kg (diamonds; n=9) or 30 mg/kg (squares; n=9) did not significantly impair reconsolidation compared to the vehicle-injected group (circles; n=10). Rats were trained with a 1.5 mA shock, reactivated a day later and tested 24 hours after reactivation. C) A 30mg/kg dose of mifepristone (squares; n=11) did not affect the reconsolidation of the conditioned response to a lower shock intensity. Rats were trained with a 0.75 mA shock, reactivated a day later and tested 24 hours after reactivation. Vehicle group (circles; n=13). D) A delayed reactivation followed by an injection of mifepristone (30 mg/kg; squares; n=8) also did not disrupt reconsolidation of the conditioned memory. Rats were trained with a 1.5 mA shock and reactivated 1 week later. They were tested 24 hours after the reactivation session. Vehicle group (circles; n=13).

reactivating the memory (McGill Figure 2D). These changes were motivated by recent evidence that the ability of mifepristone to disrupt reconsolidation of fear memory depends on the intensity and age of the memory. Mifepristone failed to block reconsolidation under these conditions. However, our colleagues at the MGH were able to show reconsolidation blockade using a 30 mg/kg dose of mifepristone. In order to determine why we did not obtain these results, we compared the fear-conditioning protocols and identified two differences – the rat supplier and the set-up of the conditioning chambers. Therefore, we are now in the process of replicating the results obtained by the MGH group using the same rat supplier and conditioning chambers.

2.1.3. <u>McLean Hospital.</u> The goal of our initial experiments was to test whether systemically injected protein synthesis inhibitor anisomycin is capable of blocking fear memory reconsolidation after retrieval. Spague-Dawley rats (250-300 g) were trained in a single-trial fear-conditioning paradigm as above. The rats were conditioned on the training day and tested at 24 h post-training in a second context. Following reactivation, rats received one injection of anisomycin (200 mg/kg, I.P.) immediately after the fear memory test. Fear memory was re-tested at 24 h post-retrieval. One hour later, the rats were sacrificed for brain slice electrophysiological analysis. At the behavioral level, we did not observe significant differences between vehicle- and anisomycin-injected rats in conditioned freezing after fear memory reactivation (P = 0.88). In parallel experiments, we examined synaptic function in thalamic input to the lateral nucleus of the amygdala (LA) in slices from behaviorally trained rats (same as in the above-described experiments) following fear memory reactivation. We found that synaptic strength, as assessed by input-output curves for AMPA receptor-mediated EPSCs, was unchanged in slices from anisomycin-injected rats, compared to vehicle-injected control rats. Membrane excitability of neurons in the lateral nucleus of the amygdala was also unaffected following fear memory reactivation in slices from anisomycin-injected rats. These results indicate that, under the present experimental conditions, systemically delivered anisomycin did not have significant effects on either fear memory reconsolidation after retrieval or synaptic strength in inputs to the amygdala providing information about the CS during auditory fear conditioning.

We next tested whether systemically-injected rapamycin (serolimus) is capable of blocking post-retrieval reconsolidation of fear memory. Rapamycin is an efficient blocker of mTOR, protein kinase that regulates protein synthesis at the translational level. In these experiments, the single-trial fear conditioning training procedures were the same as in the above-described experiments with anisomycin. The rats were conditioned in one context and tested at 24 h post-training in a different context. Following reactivation, rats received one injection of rapamycin (20 mg/kg, I.P.) immediately after the fear memory test. Fear memory was re-tested at 24 h post-retrieval. In these experiments, we tested UCSs of different intensities. Decreasing the UCS intensity to 0.6 mA was found to be optimal as rats still could be reliably conditioned, but fear responding did not reach the saturation level (as with the 1 mA shock). As shown in McLean Figure 1, we observed significant differences between vehicle- and rapamycin-injected rats in conditioned freezing after fear memory reactivation (P = 0.05). These results indicate that, under the present experimental conditions, systemically delivered rapamycin had significantly suppressed fear memory reconsolidation after retrieval.

It has been established previously that the formation of conditioned fear memory implicates long-term-potentiation (LTP) mechanisms. Consistent with the role mTOR in the induction of LTP in auditory inputs to the lateral amygdala, known to be involved in auditory fear conditioning, we found that LTP in projections from the auditory thalamus (thalamic input) and

auditory cortex (cortical input) to the LA was significantly diminished in amygdala slices treated with 2 microM rapamycin (cortical input: n = 15 neurons for vehicle-treated slices; n = 17 neurons for rapamycin-treated slices, $P < 0.02$; thalamic input: n = 13 neurons for vehicle-treated slices; n = 10 neurons for rapamycin-treated slices, $P < 0.01$). We are now continuing the experiments with rapamycin, examining further its effects on reconsolidation of conditioned fear memory and on synaptic function in thalamic and cortical inputs to the LA in slices from behaviorally trained rats (same as in the above-described experiments) following fear memory reactivation.
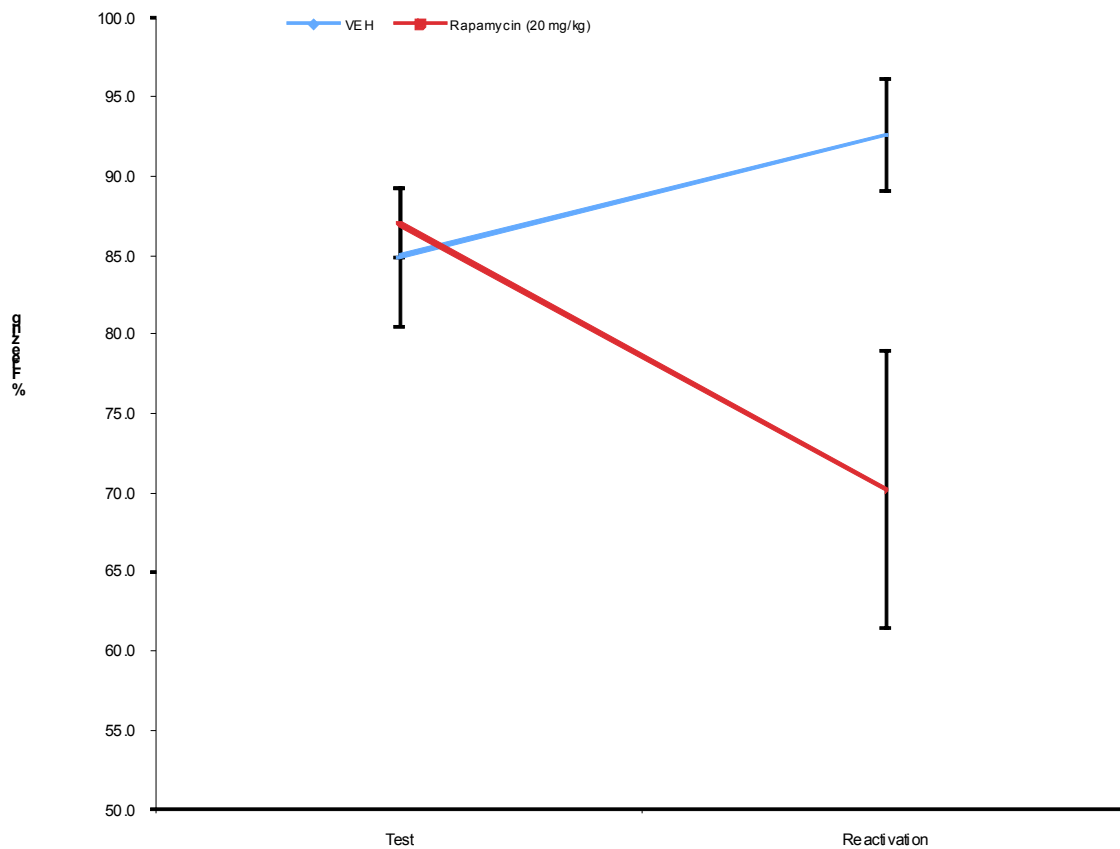

McLean Figure 1. Summary of behavioral results. The y-axis displays Day 3 freezing as percent of Day 2 freezing. VEH: Vehicle

2.4. Human work

2.4.1 <u>MGH.</u> We decided to delay initiation of our first human experiment until sufficient animal data could be obtained to guide the selection of the drug to be employed. Based upon our successful demonstration of the ability of PR mifepristone to reduce reconsolidation of a cue-conditioned fear response, we decided to perform a pilot study of PR mifepristone's ability to reduced psychophysiologic responding during traumatic imagery in trauma-exposed human subjects. We recently succeeded in obtaining an investigational new drug (IND) approval from

the FDA for this novel post-marketing application of mifepristone. We are now in the final stages of obtaining approval from the MGH's IRB. Following that, we will prepare a DOD IRB submission.

    2.4.2. <u>McGill University.</u> Despite the negative results obtain with propranolol in the animal work described above, we decided to proceed with a double-blind, randomized, placebo-controlled pilot trial of six sessions of post-reactivation propranolol for the treatment of PTSD. Several considerations motivated this decision. First, an influential article published in early 2009 succeeded in demonstrating that propranolol blocked the reconsolidation of a conditioned fear memory in normal humans, in a sense bypassing the need for further confirmatory rat studies. Second, in previously published work, we succeeded in demonstrating that a single session of propranolol following reactivation of the traumatic memory in PTSD patients significantly reduced a biological PTSD marker, viz., physiologic responding during subsequent script-driven imagery of the event. Third, during the first part of the 01 year, we completed the analysis of a previously collected data set from an open label, six session, post-reactivation propranolol case series in 32 PTSD patients.   Results from this work serve as the basis for now-proposed trial. PTSD Check List (PCL) scores are presented in the McGill-Douglas Hospital Figure 1 below.   Patients improved significantly between the pre- and post-treatment sessions ($p<.001$.) The effect size was a very large $d = 1.70$. Mean symptom improvement was 49.5%.
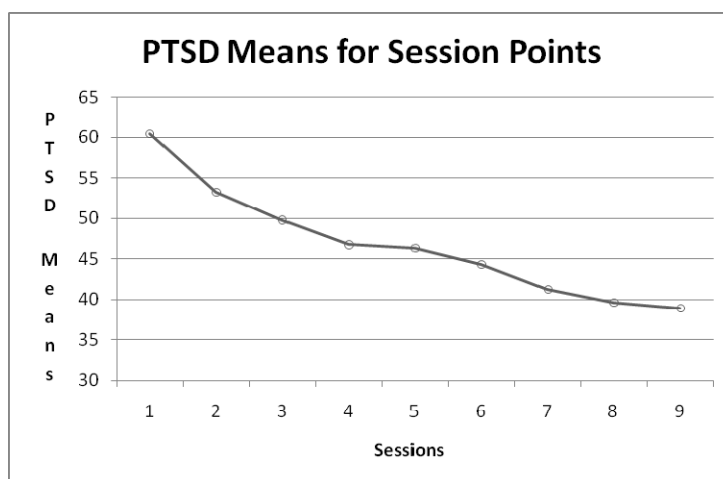
McGill-Douglas Hospital Figure 1. The y-axis displays group mean scores on the PTSD Check List. (Note that the on this instrument, the lowest possible score of 17 indicates no symptoms.)   The x-axis displays session number, as follows: 1: Pre-treatment assessment; 2: Traumatic script preparation; 3-8: PR propranolol treatment sessions (weekly); 9: Post-treatment assessment

    Approval of the presently proposed double-blind pilot trial has been obtained from the Douglas Mental Health University Institute Institutional Review Board (IRB) and Health Canada. DOD IRB approval is pending but has been delayed by several considerations. The DOD IRB required that the original French material be translated into English by a certified translator (and not by the PI even though he is perfectly fluent in English) in order to ensure a perfect match. The certified translator has now been identified and work has begun. A second obstacle was obtaining a Canadian human subject protection training certificate acceptable to the USAMRMC. Such training has finally been identified and a certificate should be issued shortly to Dr. Brunet by the Fonds de la Researche en Santé Québec (FRSQ) agency. This agency is

recognized by the tri-council of Canada and has been identified by the DOD as an acceptable institution to deliver such certification. Once those requirements are met, and we respond to the other minor issues raised by the DOD IRB, we expect to obtain ratification fairly shortly. In the meantime, all necessary procedures are in place, and staff members have been trained in their execution.

3. KEY RESEARCH ACCOMPLISHMENTS

Key accomplishments to date are the demonstration that the anti-progesterone and glucocorticoid receptor antagonist mifepristone, and the protein-synthesis inhibitor rapamycin, when administered systemically reduce reconsolidation of a cue-conditioned fear response in rats.

4. REPORTABLE OUTCOMES

The mifepristone results are being prepared for publication. Additional animals are being tested with PR rapamycin prior to considering submission for publication.

5. CONCLUSION

During the first year of this four-year project, we identified in animal work a pharmacologic agent, viz., the anti-progesterone and glucocorticoid receptor antagonist mifepristone, that is suitable for testing in humans as a novel therapeutic agent for PTSD within the framework of memory reconsolidation theory.

6. REFERENCES

None

7. APPENDICES/SUPPORTING DATA

None